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PATENT

TO ALL WHOM IT MAY CONCERN:

Be it known that we, David M. Neville, Joshua E. Scharff, and Jerry Todd Thompson, citizens of the United States of America, and Huaizhong Hu and Shenglin Ma, citizens of ..., residing respectively at 9624 Parkwood Drive, Bethesda, Maryland 20814, 4600 Harlan Street, Rockville, Maryland 20853, Parkside Apartments, Bethesda, Maryland; 4998 Battery Lane, #321, Bethesda, Maryland 20814; and 112-A North Bedford Street, Arlington, Virginia 22201, U.S.A., have invented new and useful improvements in

AN IMMUNOTOXIN WITH
IN VIVO T CELL SUPPRESSANT ACTIVITY
AND METHODS OF USE

for which the following is a specification.

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This application for letters patent claims priority under 35 U.S.C. § 119(e) of provisional patent application serial number 60/008,104, filed on October 30, 1995, having the same title of invention and the same inventors (as added by amendment to correct inventorship filed September 11, 1996) as the present application.

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membrane translocation functions (Youle and Neville (1982) *J. Clin. Biol.* 257:1598; Neville (1986) in *CRC Crit. Rev., Therap. Drug Carrier Syst.*, CRC Press Inc., 2:329; Immunotoxins, Frankel ed.(1988) Kluwer Academic Publishers). Because of this latter consideration most *in vivo* clinical studies have focused on A chain immunotoxins or immunotoxins with truncated B chains lacking the receptor binding domain. While some clinical results have been encouraging, the reproducible achievement of both goals is at present uncertain (Program and Abstracts 2nd Int. Symposium on Immunotoxins, June 1990, Lake Buena Vista, Florida).

Recently, Youle and co-workers have introduced highly efficacious holo-immunotoxins based on diphtheria toxin (DT) binding mutants (Greenfield et al. (1987) *Science* 238:536; Johnson et al. (1988) *J. Biol. Chem.* 263:1295; Johnson et al. (1989) *J. Neurosurg.* 70:240). These DT binding site mutants were equal to the wild-type immunotoxins in potency when directed at the human transferrin receptor (TFR) or human CD3, a component of the T cell receptor complex. Since the binding of the mutants was only 1/100-1/1000 of native DT, the toxin receptor appeared to be not needed along the intoxication pathway. This conclusion is limited to immunotoxins which route through CD3 and TFR, because similar immunotoxins directed at CD5 and the high-molecular weight-melanoma-associated antigen are relatively non-toxic (Neville et al. (1989) *J. Biol. Chem.* 264:14653). On the basis of data obtained with acid-cleavable conjugates which released free DT or the DT binding site mutant CRM9 in acidified endosomes, it was concluded that the DT receptor participates in the optimal intracellular routing of DT and many DT conjugates (Neville et al. (1989) *J. Biol. Chem.* 264:14653). It was also concluded that CD3 and TFR can perform the same routing function as the DT receptor, thus obviating the requirement of a DT receptor

interaction for the binding site mutant conjugates anti-CD3-CRM9 and TFR-CRM9 (Intracellular routing of ricin based immunotoxins via the ricin receptor leading to enhanced efficacy has also been reported. Youle et al. (1981) *Cell* 23:551; Marsh and Neville (1986) *Biochem.* 25:4461; Youle and Colombatti (1987) *J. Biol.Chem.* 262:4676). Since anti-CD3-CRM9 appears to achieve optimal routing with low non-target cell toxicity as judged by *in vitro* assays, the present invention relates to a method of eradicating human CD3 bearing tumors *in vivo*.

The present invention provides in one embodiment, the anti-CD3 immunotoxin scUCHT1-DT390. Routine modifications of this immunotoxin that do not significantly effect the toxicity or specificity are within the scope of the invention. The invention provides, in further embodiments, other immunotoxin constructs and methods of treating T cell leukemias or lymphomas, graft-versus-host diseases, and autoimmune diseases by administering the immunotoxin.

SUMMARY OF THE INVENTION

It is a general object of this invention to provide an immunotoxin.

It is a specific object of this invention to provide an immunotoxin.

It is a further object of the invention to provide a method of treating T cell leukemias or lymphomas.

It is a further object of the invention to provide a method of treating autoimmune diseases.

In one embodiment, the invention provides a method of treating an autoimmune disease in an animal comprising

administering to the animal an antibody-DT mutant immunotoxin which routes by the anti-CD3 pathway, or derivatives thereof, under conditions such that the autoimmune disease is treated.

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In a further embodiment, the invention provides a method of treating T cell leukemias or lymphomas in an animal comprising administering to the animal an antibody-DT mutant immunotoxin which routes by the anti-CD3 pathway, or derivatives thereof, under conditions such that the T cell leukemias or lymphomas are treated.

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Further objects and advantages of the present invention will be clear from the description that follows.

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BRIEF DESCRIPTION OF THE DRAWINGS

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Figure 1. Nude mice bg/nu/xid maintained in a semi-sterile environment are preconditioned with 400 cGy whole body ^{137}CS γ radiation on day -7. On day 0, 2.5×10^7 Jurkat cells (human T cell leukemia CD3+, CD4+, CD5+) are injected subcutaneously with 1×10^7 HT-1080 feeder cells (human sarcoma) which have received 6000 cGy. Jurkat cells were passaged every other week in mice as subcutaneous tumors and dissociated by collagenase/dispase prior to inoculation. This cell population exhibits a 40% inhibition of protein synthesis after 5 hours exposure to 10^{11}M anti-CD3-DT. Clones isolated from this population by infinite dilution exhibit varying sensitivity to anti-CD3-DT (4 less sensitive, 3 more sensitive) corresponding to a 1.5 log variation in dose response curves. Immunotoxin treatment is given by intraperitoneal injection starting on day 7 when the tumor is visibly established. Evaluation takes place on day 37.

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Figure 2 shows that the epitopes involved in human serum's inhibition of toxicity lie in the last 150 amino

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acids of DT. A schematic diagram of the DT mutants CRM9, CRM197 and MSPΔ5 is presented (A). The A- and B-subfragments and their relative size and position are shown. The filled circle represents a point mutation as described in the text. Goat (B) or human (C) serum (human serum was a pool from all samples with positive ELISA for anti-DT antibodies) was incubated with increasing molar concentrations of CRM197 (-O-), MSPΔ5 (-X-) or the B-subfragment (-Δ-) of DT for 30 minutes at room temperature. To this reaction, UCHT1-CRM9 was added to a final concentration of 1×10^{-10} M. This mixture was then diluted 10-fold onto Jurkat cells in a protein synthesis inhibition assay as described in the Materials and Methods. Immunotoxin incubated with medium only inhibited protein synthesis to 4% of controls. The results are representative of two independent assays.

Figure 3 shows that sFv-DT390 maintains specificity for the CD3 complex but is 16-fold less toxic than UCHT1-CRM9 to Jurkat cells. A) Increasing concentrations of sFv-DT390 (-Δ-) or UCHT1-CRM9 (-O-) were tested in protein synthesis inhibition assays as described in the Materials and Methods. The results are an average of four separate experiments. B) Increasing concentrations of UCHT1 antibody were mixed with a 1×10^{-10} M UCHT1-CRM9 (-O-) or 3.3×10^{-10} M sFv-DT390 (-Δ-) and then added to cells for a protein synthesis inhibition assay.

Fig. 4 shows the schematic flow sheet for generation of the single chain antibody scUCHT1 gene construct. PCR: polymerase chain reaction; L: linker; SP: signal peptide. P1 to P6, SP1, and SP2 are primers used in PCR, and listed in table 1.

Fig. 5 shows the western blotting analysis of the single chain antibody scUCHT1. scUCHT1 was immunoprecipitated, and separated on 4-20% SDS/PAGE gradient gel. After transferring to Problott™ membrane, scUCHT1 was visualized by an anti-human IgM antibody labeled with phosphatase. scUCHT1 secreted was mainly a dimeric form. Lane 1-3 representing electrophoresis under reducing conditions, and 4-6 non-reducing conditions. Lane 1 and 6 are human IgM; lane 1: IgM heavy chain. The light chain is not visible, because the anti-IgM antibody is directed at the heavy chain; lane 6: IgM pentamer is shown as indicated by the arrow. Lane 2 and 4 scUCHT1 from COS-7 cells; 3 and 5 scUCHT1 from SP2/0 cells.

Fig. 6 ^{shows} that scUCHT1 had the same specificity and affinity as its parental antibody UCHT1. In the competition assay, ¹²⁵I-UCHT1 was used as tracer in binding Jurkat cells. scUCHT1 from COS-7 (□) and SP2/0 cells (Δ), or unlabeled UCHT1 (○) with indicated concentrations were included as competitor. Results were expressed as a percentage of the ¹²⁵I-UCHT1 bound to cells in the absence of competitors.

Fig. 7 shows that scUCHT1 did not induce human T cell proliferation response. scUCHT1 from COS-7 (Δ) and SP2/0 (○) cells and UCHT1 (□) were added to human PBMCs at indicated concentrations and T cell proliferation was assayed by [3H]thymidine incorporation. UCHT1 induced a vigorous proliferation response. On the contrary, scUCHT1 had little effect at any doses.

Fig. 8 shows that scUCHT1 had little effect on TNF-α secretion, and inhibited the basal production of IFN-γ. scUCHT1 from both COS-7 (Δ) and SP2/0 (○) cells and

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UCHT1 (\square) were added to cultures of human blood mononuclear cells. Culture supernatant was harvested and used for ELISA determination of TNF- α and IFN- γ as described in materials and methods.

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- a. TNF- α secretion induced by UCHT1 and scUCHT1.
- b. IFN- γ production induced by UCHT1 and scUCHT1.

Fig. 9 is a western blot showing the secreted scUCHT1 immunotoxin.

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Fig. 10 shows a PCR amplification scheme.

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Fig. 11 shows one clone expressing the divalent immunotoxin fusion protein shown in Figure 13.

Fig. 12 shows another clone expressing a divalent immunotoxin fusion protein shown in Fig. 14.

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Fig. 13 is a schematic of a divalent fusion immunotoxin.

Fig. 14 is a schematic of a divalent fusion immunotoxin.

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Fig. 15 is a schematic of a divalent fusion immunotoxin.

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Fig. 16 shows the cloning scheme used to obtain scUCHT1 fusion protein with DTM1 and DT 483.

Fig. 17 shows the cloning scheme used to obtain scUCHT1 fusion protein with DT 390.

Fig. 18 the cloning scheme used to obtain scUCHT1 fusion protein with DT 370.

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DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to an immunotoxin. More specifically, an immunotoxin, comprising a mutant diphtheria toxin moiety linked to a single chain variable region antibody which routes by the anti-CD3 pathway is provided. The immunotoxin can be divalent. The immunotoxin can be a fusion protein produced recombinantly. The antibody moiety of the immunotoxin can comprise the human CH2 and CH3 regions. These regions can be from the antibody UCHT1 so that the antibody moiety is scUCHT1, which is a single chain CD3 antibody having human CH2 and CH3 regions and mouse variable regions as shown in the figures. These are the first instances of a sc anti-CD3 antibodies. Numerous DT mutant toxin moieties are described herein, for example DT390. Thus, as just one specific example the immunotoxin, the invention provides scUCHT1-DT390. Derivatives of this immunotoxin are designed and constructed as described herein.

The toxin moiety retains its toxic function, and membrane translocation function to the cytosol in full amounts. The loss in binding function located in the C terminus of the protein diminishes systemic toxicity by reducing binding to non-target cells. The routing function normally supplied by the toxin binding function is supplied by the targeting antibody anti-CD3. The essential routing pathway is (1) localization to coated pits for endocytosis, (2) escape from lysosomal routing, and (3) return to the plasma membrane. Any antibody which can route in this manner will be effective with the toxin moiety, irrespective of the epitope to which the antibody is directed. Thus, a wide variety of cell types can in principle be targeted. When antibodies dissociate from their receptors due to changes in receptor configuration induced in certain receptors as a consequence of endosomal acidification, they enter the lysosomal pathway. This can

be prevented or minimized by directing the antibody towards an ecto-domain epitope on the same receptor which is closer to the plasma membranes (Ruud, et al. (1989) *Scand. J. Immunol.* 29:299; Herz et al. (1990) *J. Biol. Chem.* 265:21355). Other DT binding site mutants can be used to form derivatives by changing amino acids in the C-terminus which can reduce the binding function as long as the translocation function is maintained. Specific examples are described in the Examples.

In another embodiment, the present invention relates to an anti-CD3-CRM9 immunotoxin or derivatives thereof. The design of successful derivatives of anti-CD3-CRM9 depend upon understanding how the unique concentration of anti-CD3-CRM9 achieves its biological effect.

An example of a series of derivatives which is likely to be effective are antibody-CRM9 conjugates directed at unique V α and V β gene segment products of the T cell receptor. Some of these epitopes appear to be biased towards specific autoimmune processes. Such conjugates should be useful in specific autoimmune diseases (Kappler et al. (1987) *Cell* 49:263; Urban et al. (1988) *Cell* 54:577).

Relatedly, the invention provides an anti-V β -CRM9 immunoconjugate such as anti-V β_{12} -CRM9. Also provided is an anti-V α -CRM9 immunoconjugate. Both of the conjugates can be placed in a pharmaceutically acceptable carrier for administration to a subject. Both acid-cleavable and non-cleavable protein cross-linking reagents can be used in the construction of antibody-diphtheria toxin binding-site mutant conjugates like anti-CD3-CRM9 (Neville et al. (1989) *J. Biol. Chem.* 264:14653-14661); preferred are non-cleavable crosslinkers, such as bismaleimido-hexane and m-maleimidobenzoyl-N-hydroxysuccinimide ester. The synthesis of acid-cleavable protein cross-linking reagents

based on orthoester, acetal, and ketal functionalities has been described (Srinivasachar and Neville (1989) *Biochemistry* 28:2501-2509). The unique feature of these functionalities is that their observed hydrolytic rate constants increase 10-fold for each drop in pH, a consequence of specific H_3O^+ catalysis leading to a carbonium ion intermediate (Cordes and Bull (1974) *Chem. Rev.* 74:581-603). Moreover, these functionalities are resistant to base catalysis permitting manipulation and storage at alkaline pH. The cross-linking reagents react with proteins via heterobifunctional groups (maleimide and N-hydroxysuccinimide ester) or homobifunctional groups (bis-maleimide). The maleimide cross-linking is accomplished by prior protein thiolation with iminothiolane. Cross-linked proteins exhibit first-order dissociation under acid conditions. The $t_{1/2}$ at pH 5.5 varies between 0.1 and 130 h for a series of six different cleavable cross-linkers (Srinivasachar and Neville (1989) *Biochemistry* 28:2501-2509).

The CRM9 conjugates of the invention can be expected to be effective as immunotoxins, because the relevant parameters are known. The relevant binding constants, number of receptors and translocation rates for humans have been determined and used. Binding values for anti-CD3-CRM9 for targeted and non-targeted cells *in vitro* are described above at page 2. Rates of translocation for the anti-CD3-CRM9 conjugate to targeted and non-targeted cells *in vitro* are described in references cited at page 2 (Greenfield et al. (1987) *Science* 238:536; Johnson et al. (1988) *J. Biol. Chem.* 263:1295; Johnson et al. (1989) *J. Neurosurg.* 70:240; and Neville et al. (1989) *J. Biol. Chem.* 264:14653). The rate limiting translocation rate to targeted cells *in vitro* is recited at page 5, wherein it is shown that the conjugate is translocated to about 40% of the target cells present as measured by inhibition of protein synthesis in about 40% of cells. Inhibition of

protein synthesis is complete in cells into which the conjugate translocates.

Parameters determined in *in vivo* studies in nude mice include the following: Tumor burden is described in Example 1 as a constant mass equal to 0.1% of body weight; the receptor number and variation of receptor number are described in Example 3; "favorable therapeutic margin" is defined as an *in vivo* target cell 3 log kill at 0.5 MLD (minimum lethal dose) comparison of efficacy with an established treatment of 0.5 MLD immunotoxin equivalent (group 1) to a radiation dose of 500-600 cGy (groups 8 and 9).

The parameters determined *in vitro* allowed the prediction of success in the *in vivo* nude mouse study. The prediction of *in vivo* success was verified by the data in Examples 3-4. Using the target cell number from the mouse study as being equivalent to the local T cell burden in a monkey or man successful T cell ablation and immunosuppression in monkeys could be predicted. This prediction has been verified by the monkey data in Examples 5 and 7-8. Using the same parameters, a scientist skilled in this field can make a prediction of success in humans with confidence, because these parameters have been previously shown to have predictive success.

In another embodiment, the present invention relates to a pharmaceutical composition comprising anti-CD3-DT mutant in an amount effective to treat T cell leukemias or lymphomas which carry the CD3 epitope, graft-versus-host disease or autoimmune diseases, and a pharmaceutically acceptable diluent, carrier, or excipient. One skilled in the art will appreciate that the amounts to be administered for any particular treatment protocol can readily be determined. Suitable amounts might be expected

to fall within the range of 0.01 to 1.0 mg (toxin content) per kg of body weight.

Thus, one embodiment of the invention provides a method of treating an autoimmune disease in an animal comprising administering to the animal an immunotoxin comprising a mutant diphtheria toxin moiety and an antibody moiety which routes by the anti-CD3 pathway, or derivatives thereof, under conditions such that the autoimmune disease is treated. Any of the immunotoxins described herein can be used.

The mutant diphtheria toxin moiety can be a truncated mutant, such as DT390, DT383, DT370 or other truncated mutants, as well as a full length toxin with point mutations, such as DTM1, as described in Examples 9-11. scUCHT1 fusion proteins with DTM1 and DT483 (see Fig. 16), DT390 (Fig. 17) and DT370 (Fig. 18) have been cloned and expressed in *E. coli*. The antibody moiety can be scUCHT1 or other anti-CD3 antibody having the characteristics set forth herein. Thus, one example of an immunotoxin for use in the present methods is UCHT1-DT390. The described immunotoxins can be used in all the methods of the invention.

Other examples of immunotoxins include anti-V β -CRM9 and anti-V α -CRM9. For example, the antibody-CRM9 conjugate used in any of the methods herein can be an anti-V β -CRM9 such as anti-V β_8 -CRM9. In addition, the antibody-CRM9 conjugate can be an anti-V α -CRM9. In one embodiment, the anti-V β -CRM9 is anti-V β_{12} -CRM9 and the disease is human immunodeficiency virus disease or the Acquired Immunodeficiency Syndrome (AIDS). Other V α and V β targets associated with particular autoimmune diseases exist. For example, pulmonary sarcoidosis showed increased usage of the V β_8 subset in blood and lung lymphocytes (Moller et al. (1988) *J. Clin. Invest.*

82:1183-1191). In multiple sclerosis, preferential use of the $V\beta_{5.2}$ subset in brain plaque lesions has been identified and rearrangements of $V\alpha_{1,2,7,8, \text{ and } 10}$ were also prominent (Oksenberg et al. (1993) *Nature* 362:68-70).

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A method of treating graft-versus-host disease in an animal is also provided. It comprised administering to the animal an immunotoxin comprising a diphtheria toxin binding mutant moiety and an antibody moiety which routes by the anti-CD3 pathway, or derivatives thereof under conditions such that the graft-versus-host disease is treated. Alternatively as further described, a non-toxic DT mutant such as DTM2 or CRM197 can first be administered followed by the immunotoxin

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A further embodiment of the invention provides a method of treating T cell leukemias or lymphomas in an animal comprising administering to the animal an immunotoxin comprising a non-toxic mutant of diphtheria toxin moiety and an antibody moiety which routes by the anti-CD3 pathway, or derivatives thereof, under conditions such that the T cell leukemias or lymphomas are treated. Alternatively, a further embodiment is a method of treating T cell leukemias or lymphomas in an animal comprising administering to the animal a non-toxic mutant of diphtheria toxin followed by an antibody-CRM9 conjugate which routes by the anti-CD3 pathway, or derivatives thereof, under conditions such that the T cell leukemias or lymphomas are treated.

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A method is provided for treating acquired immunodeficiency syndrome in an animal, comprising administering to the animal an immunotoxin comprising a diphtheria toxin binding mutant moiety and an antibody moiety which routes by the anti-CD3 pathway, or derivatives thereof under conditions such that the acquired immunodeficiency syndrome is treated.

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Alternatively, a method of treating acquired immunodeficiency syndrome in an animal, comprising administering to the animal a non-toxic mutant of diphtheria toxin followed by an antibody-CRM9 conjugate which routes by the anti-CD3 pathway or derivatives thereof under conditions such that the acquired immunodeficiency syndrome is treated is provided. Anti-V β_{12} is a likely conjugate for use in this method.

The non-toxic mutant of diphtheria toxin for use in the above method can be DTM2 or CRM197. DTM2 and CRM197 are non-toxic mutants of DT, having a point mutation in the enzymatic chain. However, they have the full antigenic properties of DT and CRM9, and CRM197 is used for immunization (Barbour et al. 1993. *Pediatr Infect. Dis. J.* 12:478-84). Other non-toxic DT mutants that can be used in the present method will share the characteristic of totally lacking A chain enzymatic activity. The purpose of administering the non-toxic toxin is to bind preexisting anti-CRM9 anti-DT antibodies in a subject and compete with their effect and/or induce their removal from the circulation. This substantially avoids any host immune response to the immunotoxin that might interfere with the activity of the immunotoxin.

The non-toxic DT mutant is administered prior to the administration of immunotoxin. For example, non-toxic mutant can be administered at least 5 minutes prior to the immunotoxin. A range of doses of the non-toxic mutant can be administered. For example, an approximately 10 to 100 fold excess of non-toxic mutant over the CRM9 content of the immunotoxin to be administered can be administered by I.V. route.

A H1 histamine blocking agent such as Benadryl or Tagevil can be administered I.V. prior to administering

the non-toxic mutant to minimize any possibility of an anaphylactic reaction. No evidence of anaphylactic reaction was noted in the primate experiments described in the Examples. However, the H1 histamine blocker can be administered as a precaution with no significant disadvantage.

In a further embodiment, the present invention relates to a method of treating T cell leukemias or lymphomas which carry the CD3 epitope in an animal comprising administering to an animal anti-CD3-CRM9 or derivatives thereof under conditions such that the leukemias or lymphomas regress. Appropriate concentrations and dosage unit sizes can be readily determined by one skilled in the art.

In another embodiment, the invention relates to a method of treating an immune system disorder not involving T cell proliferation which is amenable to T cell suppression. In a specific embodiment, the present invention relates to a method of treating graft-versus-host disease (GVHD) in an animal comprising administering to an animal anti-CD3-CRM9 or derivatives thereof under conditions such that the symptoms of the graft-versus-host disease improve. GVHD is a morbid complication of bone marrow transplantation which is often performed as anti-leukemia/lymphoma therapy. GVHD is caused by circulating donor T cells within the host which are acquired in bone marrow grafts unless specifically depleted prior to grafting (Gale and Butturini (1988) *Bone Marrow Transplant* 3:185; Devergie et al. (1990) *ibid* 5:379; Filipovich et al. (1987) *Transplantation* 44). Successful donor T cell depletion techniques have been associated with a higher frequency of graft rejection and leukemia relapses (Gale and Butturini (1988) *Bone Marrow Transplant* 3:185; Devergie et al. (1990) *ibid* 5:379; Filipovich et al. (1987) *Transplantation* 44). Therefore, the donor T cells

appear to aid engraftment and to provide a graft-versus-leukemia effect as well as causing GVHD. Because the T cell burden following bone marrow transplantation is low for the first 14 days (<10% of normal) the log kill of donor T cells would be proportionally enhanced (Marsh and Neville (1987) *Ann. N.Y. Acad. Sci.* 507:165; Yan et al., submitted; Gale and Butturini (1988) *Bone Marrow Transplant* 3:185; Devergie et al. (1990) *ibid* 5:379; Filipovich et al. (1987) *Transplantation* 44). It is expected that donor T cells can be eliminated at set times during the early post transplantation period using the present method. In this way the useful attributes of grafted T cells might be maximized and the harmful effects minimized.

Also provided is a method of treating graft-versus-host disease in an animal by administering to the animal a non-toxic mutant of diphtheria toxin followed by an antibody-CRM9 conjugate which routes by the anti-CD3 pathway, or derivatives thereof under conditions such that the graft-versus-host disease is treated. This method can use the antibody-CRM9 conjugates and non-toxic DT mutants described herein with the dosages and modes of administration as described herein or otherwise determined by the practitioner.

In a further embodiment, the present invention relates to a method of treating autoimmune diseases in an animal comprising administering to the animal anti-CD3-CRM9 or derivatives thereof under conditions such that the symptoms of the autoimmune disease improve. A further method of treating an autoimmune disease in an animal comprises administering to the animal a non-toxic mutant of diphtheria toxin followed by an antibody CRM9 conjugate which routes by the anti-CD3 pathway, or derivatives thereof, under conditions such that the autoimmune disease is treated.

In one preferred embodiment, AIDS is treated. Radiation induced T cell ablation with concomitant high dose zidovudine therapy followed by bone marrow transplantation has been reported to eradicate HIV-1 infection in one case (Holland et al. (1989) *Ann. Int. Med.* 111:973). Cyclophosphamide, a T cell suppressive reagent, has been shown to be beneficial in treating murine AIDS (Simard and Joliceur (1991) *Science* 251:305). Anti-CD3-CRM9 provides extensive T cell ablation without the requirement of bone marrow reconstitution.

Hemi-immunotoxins (MAbs conjugated to ricin A chain) have been used clinically as T cell suppressants for the treatment of GVHD, rheumatoid arthritis and T cell leukemia (Program and Abstracts 2nd Int. Symposium on Immunotoxins, June 1990, Lake Buena Vista, Florida; Byers et al. (1990) *Blood* 75:1426). Some positive effects have been noted. The immunotoxin described here is more toxic on a weight basis than hemi-immunotoxins, but at tolerated doses exhibits an apparent log kill of targeted cells at target cell burdens encountered clinically. This constitutes a favorable therapeutic margin. Most human sera contain anti-DT neutralizing antibodies from childhood immunization (Johnson et al. (1989) *J. Neurosurg.* 70:240). To compensate for this the therapeutic dose of anti-CD3-CRM9 can be appropriately raised without affecting the therapeutic margin. Alternatively, a non-toxic DT mutant reactive with neutralizing antisera, such as CRM197, can be administered prior to the conjugate.

The protein synthesis inhibition assay (Example 2) in the presence of human serum samples or pooled human sera becomes an important part of the evaluation of the optimal immunotoxin for the individual patient and is provide for this purpose. This assay makes routine the systematic evaluation of additional combinations of DT point

mutations and ~~carboxy~~^{CARBOXY} terminal deletions for the purpose of minimizing blockade of immunotoxin *in vivo* by anti-human antitoxin.

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The present invention will be illustrated in further detail in the following non-limiting examples.

EXAMPLE 1

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Establishment of Tumors

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The experimental design of the studies that give rise to the present invention was dictated by the goal of having an animal model as closely relevant to human *in vivo* tumor therapy as possible. In order to minimize the host killer cell immune response, bg/nu/xid strain of nude mice were used (Kamel-Reid and Dick (1988) *Science* 242:1706). The human T cell leukemia cell line, Jurkat, was chosen because of previous studies with this line and its relatively normal average complement of CD3 receptors (Preijers et al. (1988) *Scand. J. Immunol.* 27:553). The line was not cloned so that receptor variation among individual cells existed (Figure 1 legend). A scheme was developed whereby well established tumors of constant mass equal to 0.1% of body weight ($\approx 4 \times 10^7$ cells) could be achieved 7 days after inoculation of Jurkat cells (see Figure 1 and Dillman et al. (1988) *Cancer Res.* 15:5632). This required prior irradiation and inoculation with lethally irradiated helper feeder cells (see Figure 1 and Dillman et al. (1988) *Cancer Res.* 15:5632).

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EXAMPLE 2

Guinea Pig Studies

5 Immunotoxin toxicity studies were performed in guinea
pigs, an animal (like humans) with a high sensitivity to
diphtheria toxin (mice are highly resistant to diphtheria
toxin). Therapy of CRM9 conjugates was set at 1/2 the
10 guinea pig minimum lethal dose. In this study, minimum
lethal dose (MLD) is defined as the minimum tested dose
which results in both non-survivors and survivors over a 4
week evaluation period. All animals survive when a MLD is
reduced by 0.5. MLD was evaluated in guinea pigs (300-1000
15 g) by subcutaneous injection. The following MLDs were
found and are listed as μg of toxin/kg body weight; DT,
0.15; CRM9, 30; anti-CD5-DT (cleavable), 0.65; anti-CD5-
CRM9 (non-cleavable), 150. Finally, the therapeutic
efficacy of the immunotoxin treatment in producing tumor
20 regressions was compared to graded doses of whole body
irradiation which resulted in similar tumor regressions.

EXAMPLE 3

Comparison of Immunotoxins

25 Several types of immunotoxins were compared in this
study. They were synthesized as previously described by
thiolating both the monoclonal antibody moiety and the
toxin moiety and then crosslinking the bismaleimide
30 crosslinkers (Neville et al. (1989) *J. Biol. Chem.*
264:14653). Purification was performed by size exclusion
HPLC columns and fractions containing 1:1 toxin:antibody
mol ratios were isolated for these studies. Conjugates
made with an acid-labile crosslinker bismaleimidoethoxy
35 propane were compared with a non-cleavable,
bismaleimidohexane. Conjugates made with this cleavable
crosslinker have been shown to hydrolyze within the

acidifying endosome releasing free toxin moieties with half-times of hydrolysis measured at pH 5.5 of 36 min (Neville et al. (1989) *J. Biol. Chem.* 264:14653).

5 The results of this study are tabulated in Table I.
Non-treatment groups such as group 10, groups treated with
anti-CD5 immunotoxins (groups 5 and 6), and group 4
treated with a mixture of anti-CD3 and CRM9 did not show
regression. The vascularized tumor nodules that weighed
10 20 mg on day 7 grew to between 1.5 to 7.8 g on day 37 and
weighed between 7.9 and 11.6 on day 56. No late
spontaneous regressions were noted. In contrast, group 1
consisting of treatment with anti-CD3-CRM9 non-cleavable
conjugate (NC) given at 25 μ g/kg on days 7, 8, and 9 (see
15 Figure 1 time line) showed only 1 tumor out of 6 by day
37. Some of the remaining animals were subject to autopsy
and they failed to reveal residual tumor or even scarring.
Tumors identified as regressed on day 37 by superficial
inspection did not reappear during the course of the study
20 (56 days).

TABLE I. IMMUNOTOXIN AND RADIATION TREATMENT ON SUBCUTANEOUS HUMAN T CELL TUMORS (JURKAT) IN NUDE MICE

Group	Treatment	Dose (intraperitoneal)	Animals Bearing Tumors At Day 37/Group Animals	% Tumor Regressions
1	Anti-CD3-CRM9 (NC) ^a	25 µg/kg. x 3d	1/6	83
2	Anti-CD3-CRM9 (NC) Anti-CD5-CRM9 (C)	19 µg/kg. x 2d 19 µg/kg. x 2d	1/4	75
3	Anti-CD3-CRM9 (C)	25 µg/kg. x 3d	2/4	50
4	Anti-CD3+CRM9	25 µg/kg. x 3d	4/4	0
5	Anti-CD5-CRM9 (C)	25 µg/kg. x 3d	5/5	0
6	Anti-CD5-DT (NC)	25 µg/kg. x 1d	9/9	0
7	γradiation ¹³⁷ Cs	400 cGy	2/2	0
8	γradiation ¹³⁷ Cs	500 cGy	3/6	50
9	γradiation ¹³⁷ Cs	600 cGy	0/2 ^b	100
10	None		6/6	0

^aAnti-CD3 refers to the monoclonal antibody UCHT1 and was purchased from Oxoid USA, Inc. Anti-CDS refers to the monoclonal antibody T101 and was a gift from Hybritech (San Diego). NC and C refer, respectively, to non-cleavable and cleavable conjugates.

^bThese animals were evaluated on days 10 and 13 at the time of death from radiation sickness.

The cleavable crosslinker confers no therapeutic advantage to anti-CD3-CRM9 immunotoxins and may be less effective (group 3). Cleavable crosslinkers confer some advantage with anti-CD5-CRM9 conjugate in vitro (5) but had no effect in this in vivo system (group 5), and lacked significant potentiating effect when administered with anti-CD3-CRM9 (group 2). The cleavable crosslinker conferred a marked therapeutic advantage to anti-CD5 wild type toxin conjugates and tumor regressions were achieved. However, in these cases the guinea pig toxic dose was exceeded. A single dose on day 7 of cleavable anti-CD5-DT at 6 $\mu\text{g}/\text{kg}$ produced 8/10 tumor regressions while a cleavable conjugate made with an irrelevant antibody (OX8) produced no regressions (4/4). However, this dose exceeded the guinea pig MLD by 9 fold. A rescue strategy was tried in which the above conjugate dose was given intravenously followed by DT antitoxin 4 hours later (also intravenously). The 4 hr rescue could not raise the MLD above 0.65 $\mu\text{g}/\text{kg}$. The 1 hr rescue could not raise the MLD above 0.65 $\mu\text{g}/\text{kg}$. The 1 hr rescue raised the MLD to 36 $\mu\text{g}/\text{kg}$, however, there were no tumor regressions in 10 mice receiving 21.5 $\mu\text{g}/\text{kg}$ of the cleavable anti-CD5-DT conjugate.

In groups 7-9 increasing single doses of whole body radiation (102 cGy/min) were given to animals bearing 3x3x5 mm tumors. At 400 cGy no complete regressions occurred. At 500 cGy 50% complete tumor regressions occurred. At 600 cGy 100% regression was achieved as judged on day 10 and 13 when the animals died from radiation sickness. (Groups 7-9 did not receive prior radiation and tumor takes were less than 100%). It appears that the 75 $\mu\text{g}/\text{kg}$ anti-CD3-CRM9 (NC) immunotoxin is equal in therapeutic power to between 500 and 600 cGy of radiation.

EXAMPLE 4

Estimation of Cell Kill

5 The actual cell kill achieved by the radiation and
the immunotoxin can be estimated by assuming radiation
single hit inactivation kinetics along with a D_{37} value for
the radiation. A value for D_{37} of 70-80 cGy with $n = 1.2-3$
is not unreasonable for a rapidly dividing helper T cell.
10 D_{37} is the dose of radiation which reduces the fraction of
surviving cells to $1/e$ as extrapolated from the linear
portion of the log survivors vs. dose curve and n is the
intercept at 0 dose (Anderson and Warner (1976) in *Adv.*
Immunol., Academic Press Inc., 24:257). At a dose of 550
15 cGy the fraction of surviving cells is calculated to be
about 10^3 . Since a majority of tumors completely regress
at this dose we estimate that both therapies are producing
an approximate 3 log kill. (The remaining cells, $4 \times 10^7 \times 10^3$
= 4×10^4 cells apparently cannot maintain the tumor, i.e.,
20 the *in vivo* plating efficiency is low, a fairly typical
situation in the nude mouse xenograft system.) The
reliability of this 3 log kill estimate has been verified
by determining the tissue culture plating efficiency by
limiting dilution of 7 day established Jurkat tumors
25 (following dispersal) and tumors exposed 18 hours earlier
in vivo to 600 cGy. Plating efficiencies were 0.14 and
 1.4×10^4 , respectively. (Plating efficiency is the
reciprocal of the minimum average number of cells per well
which will grow to form one colony.

30

It should be emphasized that with high affinity holo-
immunotoxins the cell kill is inversely proportional to
the target cell number. This presumably occurs because
receptors are undersaturated at tolerated doses and free
35 conjugate concentration falls with increasing target cell
burden (Marsh and Neville (1987) *Ann. N.Y. Acad. Sci.*
507:165; Yan et al. (1991) *Bioconjugate Chem.* 2:207). To

put this in perspective, the tumor burden in this study is almost equal to the number of T cells in a mouse ($\approx 10^8$). It can be expected that a tolerated dose of anti-CD3-CRM9 immunotoxin can achieve an *in vivo* 3 log depletion of a normal number of CD3 positive T cells.

EXAMPLE 5

Cell Depletion in Rhesus Monkeys Induced by FN18-CRM9

FN18-CRM9 conjugate

Conjugation of anti-V β and anti-V α IgG monoclonal antibodies to CRM9 is performed by the same methods used to conjugate anti-CD3 to CRM9 using a non-cleavable linker such as bismaleimido-hexane and previously described in detail (Neville et al. (1988) *J. Biol. Chem.* 264:14653-61). The monoclonal antibody FN18 is the monkey equivalent of the human anti-CD3 (UCHT1) and is known to bind the same CD3 receptor epitopes (ϵ and γ) as bound by the human CD3 antibody and is the same isotype as the human CD3 antibody. Thus, in terms of the parameters relevant for predicting successful T cell depletion, the present CD3-CRM9 conjugate and FN18-CRM9 are expected to have the same activity.

Administration

Conjugates can be administered as an I.V. bolus in a carrier consisting of 0.1M Na₂SO₄ + 0.01M phosphate buffer, pH 7.4 plus 1 part in 50 of serum previously obtained from the subject. The dose schedule is every other or third day for 3 to 6 days. The total dose is preferably from 25 to 200 micrograms of toxin per kg of body weight.

The actual dose of FN18-CRM9 used was equal to 0.167 of the minimum lethal dose (MLD) in guinea pigs. Since

the estimation of the MLD was performed in an animal lacking an immunotoxin target cell population (guinea pigs), the true MLD of FN18-CRM9 and anti-CD3-CRM9 is expected to be higher in monkeys and humans than in guinea pigs.

T Cell Kill

Helper T cell (CD4+ cells) numbers in peripheral blood fell dramatically after the initial administration of FN18-CRM9 in two rhesus monkeys. T cell counts began to rise by day 4 (sampled just prior to the second dose of FN18-CRM9). On day 5 in monkey 8629, CD4+ cells were depressed below the limit of detection (<50 cells/mm³). Cells remained below or equal to 200/mm³ out to day 21. This low level of CD4+ cells is associated with profound immunodeficiency in humans and in monkeys (Nooij and Jonker (1987) *Eur. J. Immunol.* 17:1089-1093). The remarkable feature of this study is the long duration of helper T cell depletion (day 21) with respect to the last administration of immunotoxin (day 4) since intravenously administered immunotoxins were cleared from the vascular system with half-lives <9 hours (Rostain-Capaillon and Casellas (1990) *Cancer Research* 50:2909-2916), the effect outlasting circulating immunotoxin. This is in contrast to T cell depletion induced by unconjugated anti-CD3 antibodies (Nooij and Jonker (1987) *Eur. J. Immunol.* 17:1089-1093).

In monkey 1WS the second dose of conjugate only appeared to result in a diminished rate of CD4+ cell recovery. However, CD4+ cells were still fewer than normal at day 21. The blunted response of monkey 1WS to the second dose of immunotoxin was found to be due to a preexisting immunization of this animal to the toxin. Monkey 1WS had a significant pre-treatment anti-diphtheria toxin titer as revealed by a Western blot assay. This titer was markedly increased at day 5, indicative of a

classic secondary response. In contrast, monkey 8629 had no detectable pre-treatment titer and only a trace titer by day 5 and a moderate titer by day 28.

5 The specificity of FN18-CRM9 toward T cells can be seen by comparing the total white blood cell (WBC) count in the same two monkeys. WBCs fell, but only to 45% of baseline value on day 2 compared to 6% of baseline values for the CD4+ T cell subset. Most of the fall in WBC
10 values can be accounted for by the T cell component of the WBC population ($\approx 40\%$). However, B cells are initially depleted after FN18-CRM9 although these cells recover more quickly. FN18 is an IgG, isotype and as such is known to bind to FC_{II} receptors present on B cells and macrophages
15 with low affinity. The FN18-CRM9 depletion of B cells indicates that significant interactions between the Fc portion of the FN18 antibody and B cells is taking place.

20 The peripheral T cell depletion induced by unconjugated FN18 at a dose known to produce immunosuppression 0.2mg/kg/day (Nooij and Jonker (1987) *Eur. J. Immunol.* 17:1089-1093) was compared to the immunotoxin FN18-CRM9 administered at 1/9th the FN18 dose. Peripheral CD4+ T cell depletion is more pronounced and
25 more long-lasting with the conjugate. The demonstration that FN18-CRM9 reduces peripheral helper T cell subset (CD4+) to levels less than or equal to 200 cell/mm³ for a period as long as 21 days demonstrates that this immunotoxin and its anti-human analogs are effective
30 immunosuppressive reagents.

35 The demonstration that FN18-CRM9 is a potent agent for inducing T cell depletion in non-human primates demonstrates that an anti-human homolog of FN18-CRM9, UCHT1-CRM9 (Oxoid USA, Charlotte, NC) for example, is a potent agent for inducing T cell depletion in humans.

- The Fc binding region of anti-TCR/CD3 monoclonals may or may not be needed to induce T cell depletion when the anti-TCR/CD3 monoclonals are conjugated to CRM9. The FC_{II} binding regions can be removed, for example, by
- 5 forming the conjugates with $F(ab')_2$ derivatives as is indicated in the literature (Thorpe et al. (1985) *J. Nat'l. Cancer Inst.* 75:151-159). In addition, anti-TCR/CD3 IgA switch variants such as monoclonal antibody T3.A may be used (Ponticelli et al. (1990) *Transplantation*
 - 10 50:889-892). These avoid rapid vascular clearance characteristic of $F(ab')_2$ immunotoxins. $F(ab')_2$ and IgA switch variants of anti-TCR/CD3-CRM9 immunotoxins are therefore derivative anti-TCR/CD3 immunotoxins. These derivatives will avoid the B cell interaction noted and
 - 15 can increase specificity. However, IgG_{2a} switch variants will maximize T cell activation through the FC_I , receptor and may be useful in certain situations where T cell activation aids immunotoxin induced toxicity.
- 20 General methods to make antibodies lacking the Fc region or to make antibodies which are humanized are set forth in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1988. Thus, as used in the claims, antibody can
- 25 mean the entire antibody or any portion of the antibody sufficient for specific antigen or receptor binding.

EXAMPLE 6

- 30 Treatment of Autoimmune Diseases Using Other Antibody-CRM9
~~Conjugates~~ ^{CONJUGATES} which Route by the Anti-CD3 Pathway

- Since receptor recycling is a requirement for effective CRM9 based immunotoxins and since TCR/CD3
- 35 recycles as a unit, antibodies directed at other epitopes on TCR/CD3 will constitute effective derivatives, in particular antibodies directed at the approximately 50 V β

subset families or the approximately equal number $V\alpha$ subsets can be used to conjugate CRM9 and ablate specific $V\beta$ or $V\alpha$ subsets *in vivo*. In addition, in some cases it will be ~~desirable~~ ^{desirable} to develop specific monoclonal

5 antibodies reacting with unique rearrangements of either the $V\alpha$ or $V\beta$ subset families.

The advantage of targeting the specific $V\beta$ or $V\alpha$ subset(s) as opposed to the entire T cell population is

10 twofold: (1) Elimination of a $V\beta$ subset does not create a generalized immunodeficiency, only a hole in the immune repertoire is generated. Therefore, the ability to ward off most infections and maintain immune surveillance of most malignant transformations would remain intact. (2)

15 Immunotoxin log kill increases linearly as the target cell burden decreases, assuming dose is unchanged. A 50-fold increase in log kill can be obtained as the target is changed from the entire set of T cells to a single $V\beta$ subset. However, due to (1) the high affinity of binding

20 of these immunotoxins, (2) the very low total dose given which is below target cell receptor saturation and (3) the irreversible nature of the endocytotic process, the target cells deplete the effective dose and this depletion decreases as target burden decreases. Since the log kill

25 is exponential in effective dose, much higher increases in log kill than 50-fold on changing the target from T cells to a $V\beta$ subset can occur. The expected increase in log kill will only occur if the immunotoxin is specific for the defined target. Extraneous interactions with other

30 cell types via the antibody Fc piece is preferably eliminated.

Because HIV has been shown to preferentially infect one ($V\beta_{12}$) or a few of the 20 $V\beta$ subset families providing

35 a small T cell reservoir of HIV replication, and because HIV infection apparently involves an unknown superantigen, CRM9 based immunotoxins directed at these specific $V\beta$

subsets such as anti-V β_{12} -CRM9 can reduce the HIV virus load. In addition, total ablation of a V β subset in the presence of an endogenous superantigen can lead to long-term ablation of the subset since maturing T cells are negatively selected in the presence of endogenous superantigens. Since the specific V β subset responding to the superantigen is eliminated, infection cannot take place.

10 The two strategies that can be utilized for using anti-V β_{12} -CRM9 immunotoxins to treat HIV infections are (1) treatment depleting the susceptible V β subset to an extent where continued infection cannot be maintained and (2) treatment to the extent that all or nearly all of the V β_{12} subset is eradicated.

Anti-human V β monoclonal antibodies such as S5-11 (anti-V β_{12}) are available (T Cell Sciences, Cambridge, MA) and can be conjugated to CRM9 by standard methodologies.

20 Briefly, as in Example 5, conjugation of anti-V β and anti-V α IgG monoclonal antibodies to CRM9 is performed by the same methods used to conjugate anti-CD3 to CRM9 using a non-cleavable linker such as bismaleimido-hexane and previously described in detail (Neville et al. (1988) *J. of Biol. Chem.* 264:14653-61).

Conjugates can be administered as an I.V. bolus in a carrier consisting of 0.1M Na₂SO₄ + 0.01M phosphate buffer, pH 7.4 plus 1 part in 50 of serum previously obtained from the patient. The dose schedule is every other or third day for 3 to 6 days. The total dose is preferably from 25 to 200 micrograms of toxin per kg of body weight, but may be increased if anti-diphtheria toxin antibodies are present in the patient's sera in significant amounts.

Other V β or V α subsets which may be found to be associated with HIV infection can be treated in the same manner described herein by conjugating the CRM9 to the antibody specifically reactive with the appropriate V β or V α subset.

EXAMPLE 7

T Cell Depletion and Immunosuppression in Monkeys Using the Immunotoxin Anti-CD3-CRM9.

CRM9 is a diphtheria toxin (DT) binding site mutant and forms the basis of the anti-T cell immunotoxin anti-CD3-CRM9. This immunotoxin has been constructed against human and rhesus T cells and has shown above to kill 3 logs of human T cells in a nude mouse xenograft system. The present example demonstrates a 2 log kill of T cells in rhesus monkey lymph nodes that is also shown to produce prolongation of skin allograft rejection in monkeys.

Humans are immunized against diphtheria toxin by exposure to DPT vaccines in childhood. This long lasting immunity may interfere with the efficacy of DT based immunotoxins. Many monkeys are immunized against DT by natural exposure to toxin producing *Corynebacterium*. The present method addresses any potential interference of pre-existing DT antibodies with the activity of the present immunotoxins.

30 ELISA

ELISA assays were performed in order to determine the levels of anti-DT titers existing in 9 individuals in a population ages 27 to 55. There were 3 individuals with titers of 1:100 (low) and 6 with titers of 1:1000 (moderate).

Rhesus monkeys were screened by the same assay and a 1:1000 titered monkey was selected.

Administration of Non-Toxic Diphtheria Toxin Mutant

- 5 Monkeys were treated by I.V. route 5 min prior to the immunotoxin dose with a 100 fold excess of CRM197 over the CRM9 content of the immunotoxin to be administered. Just prior to administering CRM197, a H1 histamine blocking agent such as Benadryl or Tavegil was given I.V. to
- 10 minimize any possibility of an anaphylactic reaction (for Benadryl 4 mg/kg). No histaminic reaction was detected.

- Anti-CD3-CRM9 was given at a total dose between 0.1 and 0.2 mg/kg (toxin weight) in 3 equally divided doses
- 15 (approximately 0.033 mg/kg) on 3 consecutive days. In these monkeys, the total dose of immunotoxin was 0.1 mg/kg.

- Table I shows a comparison of the efficacy of anti-
- 20 CD3-CRM9 in monkeys by comparing the decrease in the lymph node T/B cell ratio (a measure of lymph node T cell depletion) and the immunosuppressive effect of the immunotoxin as judged by prolongation of mismatched skin graft survival. Effects on the survival of skin grafts is
- 25 a clear indicator of the general effect a given treatment has on the subject's immune system.

- The monkey with the preexisting anti-DT titer that was pretreated with CRM197 shows the same level of T/B
- 30 cell inversion as in the negative titered monkey. Skin graft survival was significantly prolonged over the titered monkey treated without CRM197. The failure to achieve a prolongation of graft survival equal to the negatively titered monkey is likely due to the lower
- 35 weight of this monkey which causes T cells to repopulate faster, in this case 3-4 days faster, due to the larger thymic T cell precursor pool in younger animals. Age

related effects such as these can be compensated for by modification of dosage levels and timing of administration.

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TABLE II. Efficacy of Anti-CD3-CRM9 With and Without CRM197 In Rhesus Monkeys
With Positive and Negative Anti-Diphtheria Toxin Titers.

Monkey	Weight kg	Anti-DT Titer	Treatment	Post Treatment* Lymphnode T/B Cell Ratio	Day(s) of Skin Graft Survival
historical controls	4-7	N/A	None	2.1-2.4 ⁺	9.5±08 ^s
B65	5.1	neg	anti-CD3	1.8	12, 12
8838	5.1	neg	anti-CD3-CRM9	0.14 ^{xx}	19, 20
M93	5.1	1:1000	anti-CD3-CRM9	0.57	11, 12
C81	1.0	1:1000	CRM197 + anti-CD3-CRM9	0.20	14, 15

* All monkeys received the same dose of immunotoxin 0.1 mg/kg total in divided doses on day 0, 1 and 2. Lymph node sampled on day 3. CRM197 when given in 100 fold excess over CRM9 content.

+ In this study untreated animals show this lymph node T/B ratio

\$ Historical controls at TNO, Rijswijk

xx Anti-CD3 given at the same mol. dose as anti-CD3-CRM9

EXAMPLE 8

Immunotoxin UCHT1-CRM9 for the Treatment of Steroid
Resistant Graft-Versus-Host Disease

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Treatment protocols for this type of disease can be expected to last a year, with Patients being followed for at least 5 years.

10 Characterization of UCHT1-CRM9 and CRM197

UCHT1-CRM9 is a covalent 1:1 conjugate of anti-human CD3 IgG1 monoclonal antibody and CRM9. The conjugate is synthesized, purified, sterile filtered and assayed for concentration, biological efficacy toward target cells and non-target cell toxicity by standardized culture assays. The method of synthesis, purification assay are identical to that used for FNI8-CRM9 which was used in the pre-clinical monkey studies described in Examples 5 - 7.

CRM9 and CRM197 are produced by the Biotechnology Unit, NIH and purified by the Cooperating Facility. UCHT1 is produced in mouse ascites fluid and is purified by affinity chromatography over Protein A Sepharose. The synthesis, purification and storage of UCHT1-CRM9 is performed in a dedicated secure area. UCHT1-CRM9 is purified in 2 mg lots which are pooled and stored at 4°C. Shelf life is documented to be five months at full biological potency but does not exceed 4 months for this study. Preferably, most of the immunotoxin is used within 3 months of synthesis.

Patient Population

The patient population consists of individuals suffering from steroid resistant GVHD whose prognosis is poor. Patients are assayed for anti-CRM9 (anti-DT) titers and antibodies to murine immunoglobulin. Patients having anti-CRM9 titers of 1:1000 and below are treated according

to the present protocol. Patients who have a history of receiving murine immunoglobulins or who exhibit positive anti-Ig titers may require special consideration.

5 Dosage of CRM9 Immunotoxin and Non-Toxic Mutant

UCHT1-CRM9 is administered at a dose which is 1/10 or less of the estimated minimum lethal dose (MLD) in a T lymphopenic patient. The MLD is expected to be at least 0.15 mg/kg (CRM9 content) based on the MLD of 0.15 mg/kg of IgG1-CRM9 in guinea pigs which lack a target cell population for the IgG1. (The presence of target cells in humans raises the MLD by providing a sink for the immunotoxin.) The optimal dose schedule has been found in monkeys to be administration on 3 consecutive days in 3 equally divided doses, and this schedule can be used throughout the treatment period. This permits administration of the total dose before any rise in pre-existing antitoxin titers due to a secondary response. In addition, the initial repopulation from the thymus is also eliminated, thus, further lowering the total T lymphocyte pool. Therefore, a total of 0.0125 mg/kg in three equally divided doses is given to the patient. This dose does induce T cell depletion in monkeys so that monitoring of T cell subsets and signs and symptoms of GVHD is relevant at the lowest dose. For the administration of this dose patients with anti-CRM9 titers of 1:100 or less will be treated. This permits pretreatment doses of CRM197 at 0.33 mg/kg or 1/10 the dose easily tolerated in monkeys. A second dosage group can include patients selected for antitoxin titers of 1:330 or less to whom CRM197 will be given at 1.0 mg/kg. A third dosage group can include patients with 1:1000 antitoxin titers or less will be given CRM197 at 3.3 mg/kg, a dose expected to be tolerable in humans, because it is easily tolerated by monkeys (see Example 7). The monkey MLD data should be very similar to humans on a per weight basis. However, GVHD patients are expected to be

more like guinea pigs, because they have a smaller target cell population compared to non-GVHD patients.

Dose escalation can be tested by increasing the dose by a factor of 1.5. The following table exemplifies such a dose escalation test. For example three patients are used in each dosage group. There is a 3 to 4 week delay between each patient so that any late toxicity is detected before a dosage group is completed:

Patient #	CRM9 Dose each day mg/kg	Total Dose mg/kg	Week ending
1,2,3	0.00417	0.0125	12
4,5,6	0.00636	0.019	24
7,8,9	0.0083	0.028	36
10,11,12	0.0125	0.042	48

Assuming each patient weighs on the average 70 kg, the first dosage group will consume 2.6 mg of the CRM9 immunotoxin, and will be supplied as a pool of two 2 mg batches. The second group will consume 3.9 mg and will also be supplied as 2 pooled batches. The third group will require 5.9 mg and will be supplied as three pooled batches. The fourth group will require 8.9 mg and will be supplied as three pooled batches and an additional two pooled batches.

10

Administration

Prior to administering CRM197 a H1 histamine blocking agent such as Benadryl or Tavegil is given I.V. to minimize any possibility of an anaphylactic reaction (for Benadryl 4 mg/kg). The CRM197 is administered I.V. in a 5 mg/ml sterile filtered solution in phosphate buffered saline pH 7.4 (PBS) over a 5 min time period. The immunotoxin is then given I.V. at 0.2 mg/ml over 2 min

time period in a sterile filtered solution of 0.90mM sodium sulfate and 10mM sodium phosphate pH 7.4.

Measurements of Biological Parameters

- 5 The following parameters can be measured at various intervals during treatment (as exemplified by the schedule below):

- A Cytokines, TNF alpha, gamma IFN, IL-6
- B Routine clinical chemistries
- 10 C WBC, Hct, diff; lymphocyte subsets CD3, CD4, CD8, CD2, CD16, CD20
- D Body Weight
- E Immune function assays. ELISA assays of serum to monitor antibody responses to UCHT1 (primary response) and
- 15 CRM9 (secondary response). ELISA assays to monitor antibody responses to polio and DPT reimmunizations done at 1 year following bone marrow transplantation.

	(before IT)Day 0	A,B,C,D,E	Also A 2 hrs post
20	Day 1	A,C,D	
	Day 2	A,C,D	
	Day 3	A,B,C,D	
	Day 4	C,D	
	Day 7	A,C,D	
25	Day 10	B,C	
	Day 14	A,C,D	
	Day 21	C,D	
	Day 28	A,B,C,D,E	
	Day 45	C,D	
30	Day 60,	B,C,D,E	

EXAMPLE 9

- 35 An anti-CD3 single-chain immunotoxin with a truncated diphtheria toxin ~~antibodies inhibit humoral by pre~~existing

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The present Example examines the effect of human serum with pre-existing anti-DT antibodies on the toxicity of UCHT1-CRM9, an immunotoxin directed against CD3 molecules on T-lymphocytes. Sera with detectable anti-DT antibodies at 1:100 or greater dilutions inhibited the immunotoxin toxicity. Experiments with radiolabeled-UCHT1-CRM9 indicate that anti-DT antibodies partially block its binding to the cell surface as well as inhibit the translocation from the endosome to the cytosol. The inhibitory effect could be adsorbed using a full-length DT mutant or B-subfragment. A C-terminal truncation mutant could not adsorb the inhibitory effect, suggesting that the last 150 amino acids contain the epitope(s) recognized by the inhibitory antibodies.

Therefore, an anti-CD3 single-chain immunotoxin, sFv-DT390, was made with a truncated DT. The IC_{50} of sFv-DT390 was 4.8×10^{-11} M, 1/16 the potency of the divalent UCHT1-CRM9. More importantly, sFv-DT390 toxicity was only slightly affected by the anti-DT antibodies in human sera. "sFv" and "scUCHT1" both are ^{single} single-chain antibodies containing the variable region.

Mutated full-length and truncated diphtheria toxin (DT) molecules are used for making immunotoxins. These immunotoxins show strong cytotoxic effects to their target cells, and some of them have already been used in clinical trials (1-7).] Previously, an immunotoxin directed against the CD3e molecule of the T-cell receptor complex, a pan T-cell marker was constructed. This construct is made with a monoclonal antibody of mouse-origin, UCHT1, and a binding site mutant of diphtheria toxin (DT), CRM9 (8). The immunotoxin, UCHT1-CRM9, is capable of regressing established xenografted human T-cell (Jurkat) tumors in nude mice (9). A rhesus monkey analog of UCHT1-CRM9,

FN18-CRM9 was capable of not only depleting circulating T-cells but also depleting resident T-cells in the lymph nodes¹. This immunotoxin also delayed skin allograft rejection as compared to antibody treatment and non-treatment controls. FN18-CRM9 has also been used as an adjunct in inducing tolerance to mismatched kidney transplants (24).

In contrast with ricin and *Pseudomonas* exotoxin (PE) based immunotoxins, there is a potential problem using UCHT1-CRM9, or other DT-based immunotoxins, in the treatment of human diseases. Most people have been immunized against DT. Therefore these people have a pre-existing anti-DT antibody titer which could potentially inhibit or alter the efficacy of these immunotoxins. This limitation also occurred in rhesus monkey studies. FN18-CRM9 could deplete T cells in the blood, but to a much lesser extent in animals with anti-DT antibodies, and the T cells repopulated several days earlier compared to those monkeys without anti-DT titers. In order to overcome this antibody mediated inhibition, the first examination of the effect and the mechanism of human sera containing anti-DT antibodies on UCHT1-CRM9 toxicity was done.

25

A DT point-mutant, a truncation mutant and DT-subfragments were used in an attempt to neutralize the anti-DT effect in human sera. Based on the neutralization data, a single-chain immunotoxin was constructed with a C-terminal deletion mutant of DT which is expected to bypass the inhibitory effect of the pre-existing anti-DT antibodies.

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Inhibition assays were performed as described (12) with the following modifications. Immunotoxins were incubated for 30 minutes with the indicated serum sample or leucine free medium at room temperature prior to addition to cells. In some experiments the serum was pre-incubated for 30 minutes with an adsorbing molecule at the given concentrations to bind the antibodies. The immunotoxin/serum mixture was incubated with Jurkat cells (5×10^4 cells/well in 96 well plate) for 20 hours. A 1 hour pulse of [^3H]-leucine ($4.5 \mu\text{Ci/ml}$) was given before cells were collected onto filters with a Skatron harvester. Samples were counted in a Beckman scintillation counter. Each experiment was performed in 3 replicates. Results were calculated into a mean value, and recorded as a percentage of control cells.

Anti-DT antibodies were detected in human serum by
35 ELISA. CRM9 (10 µg/ml) was adsorbed to Costar 96-well

EIA/RIA flat bottom plates (Costar, Cambridge, MA, USA) for 2 hours and then washed in phosphate buffered saline (PBS) containing 0.1% Tween 20. Each well was then incubated with PBS containing 3% gelatin to prevent non-specific binding of antibodies to the plastic. Serum samples were diluted in PBS containing 0.1% Tween 20 and 0.3% gelatin prior to addition to the plate. After 1 hour incubation, the wells were washed as above, and incubated for an additional hour with protein A/G-alkaline phosphatase (1:5,000; Pierce, Rockford, IL, USA). Wells were washed, and phosphatase substrate (Pierce) was added following the manufacturer's directions. After 30 minutes color development was stopped with NaOH and the optical density (OD) was measured with a kinetic microplate reader (Molecular Devices Corporation, Palo Alto, CA, USA). Each sample was performed in triplicate. Results are presented as O.D. values and antibody titers.

Endocytosis assay.

UCHT1-CRM9 was iodinated using the Bolton-Hunter reagent (NEN Dupont, Wilmington, DE, USA) as described (16). Jurkat cells were washed twice with binding medium (RPMI 1640 supplemented with 0.2% bovine serum albumin, 10 mM Hepes (pH 7.4) and without sodium bicarbonate). Cells (1.5×10^6) were incubated for 2 hours on ice with ^{125}I -UCHT1-CRM9 (1×10^{-9} M) that had been pre-incubated with serum or binding medium. Unbound antibody was removed by washing the cells twice in PBS (pH 7.4) with centrifugation and resuspension. Duplicate samples were incubated for 30 minutes on ice or at 37°C . One sample from each temperature point was centrifuged at $800 \times g$ to separate the total cell associated (pellet) from the exocytosed or dissociated counts (supernatant). Both fractions were counted in a Beckman γ -counter. To determine the amount of internalized immunotoxin, cells

from the second sample at each temperature were incubated in low pH medium (binding medium containing 10 mM morpholinoethanesulfonic acid, all of which was titrated to pH 2.0 with HCl) for 5 minutes to dissociate the surface bound ^{125}I -immunotoxin (17). Samples were centrifuged at 800 x g to separate the internalized (pellet) from the membrane bound (supernatant). Both fractions were counted in a Beckman γ -counter (Beckman, Fullerton, CA, USA).

10

Serum with anti-DT antibodies inhibits UCHT1-CRM9 toxicity.

Since humans are immunized against DT, the presence of anti-DT antibodies in the serum was determined by ELISA (Table 3). In a limited sample population, 80% of the serum samples had an anti-DT antibody titer of 1:100 or above. The vaccination status of the donors was not available. To determine the effect of these antibodies on UCHT1-CRM9 toxicity, the immunotoxin was pre-incubated with different concentrations of serum and the toxicity of the mixture was assayed (Table 3). Serum samples without a significant ELISA O.D. (2 fold above background) were incapable of affecting UCHT1-CRM9 toxicity at high concentrations of serum (1:10). However, serum samples with a positive ELISA result could neutralize the cytotoxic effect at 1:10 dilution, and those with a high ELISA O.D. (7-11 fold above background) inhibited toxicity even at a 1:100 dilution. Similar results were seen in assays conducted with monkey serum samples.

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Sera do not inhibit endocytosis of UCHT1-CRM9.

The inhibitory effect of serum on UCHT1-CRM9 toxicity could be due to prevention of the immunotoxin binding to the cell surface or the endocytosis of UCHT1-CRM9 into the cell. Endocytosis assays were conducted using ^{125}I -UCHT1-CRM9 to determine if either of these processes were affected by anti-DT antibodies present in sera. The results indicate that the presence of serum (goat anti-DT or human) reduces as much as 80% of the immunotoxin counts binding to the cell surface (Table 4). While this is a significant reduction in binding, limiting 90% of input immunotoxin (one log less UCHT1-CRM9) in toxicity assays reduces protein synthesis to <25% of controls (see Figure 3). In contrast, the inhibitory effect of serum containing anti-DT antibodies is 100%. Therefore the effect of the anti-DT antibodies is not all at the level of inhibition of binding to the cell surface. The pre-incubation of ^{125}I -UCHT1-CRM9 for 2 hours on ice and subsequent washing at room temperature ^{resulted} ~~resulted~~ in 18 to 25% of the total cell associated counts internalized (Table 4). After incubation for 30 minutes at 37°C, there is a doubling of internalized counts both with and without serum, indicating that the same percentage of labeled immunotoxin is endocytosed. The identical dilutions of serum were incubated with non-labeled UCHT1-CRM9 and used in protein synthesis inhibition assays. The results demonstrate that the ratio of immunotoxin to serum used was capable of completely inhibiting the toxicity (Table 4), although the endocytosis of UCHT1-CRM9 was not affected.

Table 4. Inhibition of UCHT1-CRM9 toxicity by serum does not correlate with inhibition of endocytosis.

Serum Sample	Time (37°C)	% Bound	% of Bound internalized	Protein Synthesis (% Control)
-	0	100	23.6.	N.D. ^a
-	30	100	58.8	3 ± 1
Human	0	20	18.1	N.D. ^a
Human	30	19	35.9	105 ± 5
-	0	100	25.3	N.D. ^a
-	30	100	54.0	3 ± 1
Goat	0	37	24.4	N.D. ^a
Goat	30	33	50.7	92 ± 14

[²⁵¹I]-UCHT1-CRM9 (2 x 10⁻⁹M) was incubated with medium or anti-DT serum (1:4 dilution of human sample 10010 or a 1:1,000 dilution of goat serum; Table 3) for 30 minutes at room temperature. This mixture was added to Jurkat cells (1.5 X 10⁶) for 2 hours on ice (final concentration of [²⁵¹I]-UCHT1-CRM9 was 1 x 10⁻¹⁰). The cells were then washed and endocytosis assays performed as described in Materials and Methods. The % Bound value represents the cell associated counts divided by the cell associated counts divided by the cell associated counts without serum. Non-labeled UCHT1-CRM9 was incubated with the above dilutions of sera and the resulting mixture was used in protein synthesis inhibition assays. the results shown are representative of two independent assays.

n.d.: non done.

00329955 : 000399

The inhibitory effect of anti-DT antibodies can be removed by adsorption.

To prevent the inhibitory effect of serum as well as gain insight into the mechanism by which serum inhibits toxicity, experiments were designed to adsorb the protective anti-DT antibodies from the serum. The serum (a pool of all human sera with positive anti-DT ELISA or goat anti-DT) was pre-incubated for 30 minutes with increasing concentrations of CRM197 (an A-chain mutant of DT with no enzymatic activity), MSPA5 (a truncation mutant missing the last 150 amino acids) and the purified A- and B-subfragments of DT (Figure 2A). The adsorbed serum was then incubated with UCHT1-CRM9 in protein synthesis inhibition assays. CRM197, the full length DT-like construct, was capable of completely adsorbing the protective antibodies from both goat (Figure 2B) and pooled human serum (Figure 2C). The B-subfragment of DT is also capable of complete adsorption, however ~100 fold more is required. The A-subfragment of DT had little or no effect on either serum, although the serum samples were demonstrated to contain antibodies reactive to both the A- and the B-subfragments by Western Blot analysis. Of interest were the results seen with MSPA5, the truncation mutant. Adsorption of goat serum with MSPA5 gave a dose dependent removal of the serum's protecting effect (Figure 2B). However, this adsorption could not bring toxicity down to levels obtained when CRM197 or the B-subfragment was used.

In contrast to the results observed with the goat serum, MSPA5 had little effect on pooled human serum (Figure 2C). These results suggest that the pre-existing anti-DT antibodies important for the protecting effect in human serum are mainly directed against the last 150 amino acids of DT.

in human sera.

Having observed that the epitope(s) recognized by the antibodies important for protection lay in the C-terminal 150 amino acids, a single-chain immunotoxin was generated with the first 390 amino acids (out of 535) of DT. Position 390 was chosen for 2 reasons: first, the 3 dimensional structure of DT suggested that this position was an external point on the molecule away from the enzymatic domain (18), and second, fusion toxins have been generated with longer DT subfragments with no reports of serum effects (19). The DNA encoding the first 390 amino acids of DT was ligated to DNA encoding the anti-CD3esFv (V_L linked to V_H using a ^{(Gly₄Ser)₃} linker sequence). The predicted molecular weight for the fusion protein is 71,000 Daltons and has been confirmed by Western Blot analysis of both *in vitro* transcribed and translated protein as well as protein isolated from *E. coli* using goat anti-DT antibodies. The toxicity of sFv-DT390 protein, isolated from *E. coli* strain BL21/DE3, was compared to UCHT1-CRM9 in protein synthesis inhibition assays (Figure 3A). The IC_{50} (concentration required to inhibit protein synthesis to 50% of controls) of sFv-DT390 was 4.8×10^{-11} M compared to 2.9×10^{-12} M for UCHT1-CRM9, a 16-fold difference. To demonstrate the specificity of the sFv-DT390 construct, competition experiments were performed using increasing concentrations of UCHT1 antibody as competitor (Figure 3B). The results showed that approximately 1/8 antibody is needed to compete the sFv-DT390 toxicity to 50% as compared to UCHT1-CRM9. The antibody was capable of totally competing toxicity of both constructs thereby showing their specificity. The immunotoxins were then subjected to protein synthesis assays in the presence of increasing dilutions of serum (Table 5).

UCHT1-CRM9 toxicity was completely inhibited with a 1:10 dilution of the human sera but at a 1:100 dilution toxicity was equivalent to controls without serum. In contrast, the sFv-DT390 immunotoxin is only partially inhibited with the 1:10 dilution of the human sera and the 1:100 dilution no effect on the toxicity. Both immunotoxins are completely inhibited by goat anti-DT serum (1:1,000 dilution). These results indicate that the sFv-DT390 immunotoxin partially evades the pre-existing anti-DT antibodies present in most human sera.

These results indicate that the pre-existing anti-DT antibodies present in human serum inhibit the toxicity of the immunotoxin UCHT1-CRM9. This inhibition of toxicity was also observed with goat anti-DT serum, however less goat serum was needed to completely inhibit toxicity. The experiments were designed in such a way to mimic the *in vivo* situation. The peak concentration of circulating immunotoxin currently being tested in animal models is 1×10^{-9} M. The immunotoxin concentration incubated with the 1:10 dilution of human serum was 1×10^{-10} M, thus approximating *in vivo* conditions. The inhibition of toxicity correlates with the serum antibody levels as determined by ELISA (Table 4), indicating that sera with higher anti-DT titers have a stronger inhibitory effect. Similarly, the goat anti-DT serum which gave the highest ELISA value could be diluted 10,000 times and still completely inhibited UCHT1-CRM9 toxicity. Since this correlation exists, there is no indication that any other component of the serum inhibits the toxicity of UCHT1-CRM9.

Furthermore, the data show that a titer of 1:100 dilution is necessary for an inhibition of the immunotoxin toxicity. A construct in which the first 486 amino acids

of DT were fused to interleukin-2, DAB₄₈₆IL-2, was used in lymphoid malignancy patients. A partial response to DAB₄₈₆IL-2 was observed in several patients who had a anti-DT titer below 1:100 dilution prior to the treatment.

5

Intoxication of cells by immunotoxins can be subdivided into four general stages: 1) specific binding to the cell surface, 2) endocytosis into the cell, 3) translocation of enzymatic domain of the toxin out of the endosome and 4) enzymatic inactivation of the target molecule. The results presented indicate that, while the amount of immunotoxin reaching the cell surface is lower in the presence of serum, the same percentage of bound immunotoxin is endocytosed. Taking into account the reduced amount of immunotoxin bound to the cell, the amount of endocytosed immunotoxin should intoxicate the cells to below 25% of controls. However, the immunotoxin had no effect on protein synthesis in the presence of serum containing anti-DT antibodies. Since the A-subfragment of DT could not adsorb the protective effect of serum while the B-subfragment could, the effect of serum is not likely to be at the level of inhibiting enzymatic activity of the toxin. Therefore, the anti-DT antibodies probably affect the translocation of the A-subfragment into the cytosol.

CRM197, B-subfragment, and MSPA5 could adsorb the protecting anti-DT antibodies from the goat and rhesus monkey sera. However, among the 3 DT mutants, MSPA5 could not prevent the UCHT1-CRM9 toxicity in the presence of the human sera, showing a difference in the anti-DT antibody repertoire among humans, goat and rhesus monkeys. This difference does not seem to be due to immunization routes, because monkeys used in the present study were not immunized for DT and presumably acquire the antibodies

after a natural infection with toxigenic strains of *C. diphtheriae*. There have been reports showing that rhesus monkeys and humans shared a similar antibody repertoire (21), but the present results suggest that the effect of antibodies from the host for whom immunotoxin treatment is intended should be useful.

To overcome the blocking effect of the pre-existing anti-DT antibodies in human sera, there are basically two pathways existing. One is to neutralize the antibodies with non-toxic DT mutants, and the other is to modify the DT structure used for making immunotoxin (3). The antibody neutralization pathway has been tested in monkey studies of FN18-CRM9 treatment as described above.

The present results showed that although antibodies against both A- and B-subfragments existed in human sera, MSP5 could not neutralize the pre-existing protective anti-DT antibodies, and therefore could not prevent the inhibition of the cytotoxicity of UCHT1-CRM9. However, it did block the inhibitory effect of the goat and monkey sera. This prompted the construction of the present recombinant immunotoxin, sFv-DT390. The IC_{50} of sFv-DT390 is 4.8×10^{-11} M, 1/16 as potent as UCHT1-CRM9. Like many other single-chain constructs, sFv-DT390 is monovalent as compared to immunotoxins generated with full length, bivalent antibodies. The reduced toxicity in sFv-DT390 could be explained primarily on this affinity difference. Immunotoxins generated with purified F(ab)' fragments of antibodies also show an *in vitro* loss in toxicity (generally a 1.5 log difference) when compared to their counterparts generated with full length antibodies (22). The toxicity of sFv-DT390 is comparable to that reported for DAB486IL-2 (23). From the present data some advantages of sFv-DT390 are expected. First, sFv-DT390 is

only 1/3 of the molecular weight of UCHT1-CRM9. The molar concentration of sFv-DT390 will be 3 times higher than that of UCHT1-CRM9 if the same amount is given (for example, 0.2 mg/kg). Therefore, their difference in

5 potency could be reduced to approximately 5 times.

Second, in an *in vitro* experiment (Table 5), the same molar concentration of sFv-DT390 and UCHT1-CRM9 was used for serum inhibition test, although the former is only 1/16 potent compared to the latter. The pre-existing

10 anti-DT antibodies in human sera could only partially block the toxicity of sFv-DT390 while the effect of UCHT1-CRM9 was completely blocked. Thus, sFv-DT390 is expected to bypass the anti-DT antibodies in *in vivo* situations while UCHT1-CRM9 cannot. Third, sFv-DT390

15 contains only the variable region of UCHT1, and is expected to have less immunogenicity in human anti-mouse antibody (HAMA) responses than the native murine antibody UCHT1. Finally, the production cost of sFv-DT390 is much lower than that of UCHT1-CRM9. Based on these reasons,

20 sFv-DT390, or others with similar properties, are expected to be useful in the treatment of T-cell mediated diseases in humans, especially in anti-DT positive individuals and in patients who need repeated ^{treatment} ~~treatments~~. To obtain evidence supporting this assumption, it is only necessary

25 to construct a rhesus monkey analog of sFv-DT390, and test it in monkey models as described in previous examples.

Table 5: Anti-DT antibodies present in human sera have reduced effect on sFv-DT390 toxicity.

Serum Sample	ELISA value (\pm S.D.)	Protein synthesis (% Control)				
		UchT1CRM9		sFv-DT390		
		1:10	1:10 ²	1:10 ³	1:10	1:10 ²
10012	0.491 \pm 0.025	119 \pm 24	8 \pm 2	ND*	47 \pm 9	21 \pm 8
Pooled	0.331 \pm 0.015	108 \pm 37	7 \pm 1	ND*	49 \pm 7	16 \pm 7
Goat	1.450 \pm 0.013	ND	ND	94 \pm 21	ND	ND
						8 \pm 11

*Not done

UCHT1CRM9 or sFv-DT390 (2×10^{-9} M) was incubated with the indicated dilutions of serum for 30 min. The mixture was then added to cells as described under "Materials and Methods." The final concentration of immunotoxin on cells was 1×10^{-10} M. Four replicates were performed for each sample. Data are presented as a mean value \pm S.D. in percentage of the control counts. UCHT1-CRM9 inhibited protein synthesis to 5% of controls while the sFv-DT390 inhibited protein synthesis to 18% of controls. The ELISA value was determined using a 1:100 dilution of serum. The results are representative of two independent experiments.

Expression and Characterization of A Divalent Chimeric Anti-human CD3 Single Chain Antibody

Murine anti-CD3 monoclonal antibodies (mAbs) are used in clinical practice for immunosuppression. However, there are two major drawbacks of this treatment: the associated cytokine release syndrome and human anti-mouse antibody response. To overcome these side effects, a chimeric anti-human CD3 single chain antibody, scUCHT1 was generated. It is an IgM variant of the UCHT1 described in Example 9. scUCHT1 consists of the light and heavy variable chain binding domains of UCHT1 and a human IgM Fc region (CH₂ to CH₄). The method used was reported by Shu et al. [37] and is further described below. The following data show that the engineered chimeric anti-CD3 single chain antibody (scUCHT1) will be useful in clinical immunosuppressive treatment.

Oligonucleotide primers and DNA amplification.

Primers used for the antibody engineering are listed in Table 6, and the primer sequences are based on published data [13]. The procedures of cloning scUCHT1 is schematically depicted in Fig. 4. mRNA isolated from UCHT1 hybridoma cells (provided by Dr. P. C. Beverley, Imperial Cancer Research Fund, London) was reverse transcribed into cDNA. The V_L and V_H regions of UCHT1 were amplified with polymerase chain reaction (PCR) from the cDNA using primer pairs P1, P2 and P3, P4 respectively. Primers P2 and P3 have a 25 bp complementary overlap and each encoded a part of a linker peptide (Gly₄Ser)₃. The single chain variable fragment (V_L -linker- V_H) was created by recombinant amplification of V_L and V_H using primers P1 and P4. A mouse kappa chain signal sequence was added at the V_L 5'-end by PCR, first with primers SP2 and P4, and then with primers SP1 and P4. The human IgM Fc region (CH_2 to CH_4) was amplified from the plasmid pBlue-huIgM (kindly provided by Dr. S. V. S. Kashmiri, National Cancer Institute, Bethesda). This gene fragment was about 1.8 kb. The V_L -linker- V_H - CH_2 region which is important for antigen recognition was confirmed by sequence analysis. Finally, the single chain variable fragment and the human IgM Fc region were cloned into plasmid pBK/CMV (Stratagene, La Jolla, CA, USA). Using the generated pBK/scUCHT1 plasmid as template, an *in vitro* transcription-translation assay yielded a product of 75 kDa, the expected size.



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Table 6. Sequences of oligonucleotide primers used for PCR amplification

Sequence ID Number	5'	Sequence	3'	Primers	RE sites
SEQ ID NO. 7	GACATCCAGATGACCCAGACC			P1 (UCHT1 VL5)	
SEQ ID NO. 8	CCTCCGAGCCACCGCCTCGCTCGCCTCCCTTTTA TCTCCAGCTTG(T)GTC(G)CC			P2 (UCHT1 VL3)	
SEQ ID NO. 9	GCAGCGGAGGCGGTGGCTCGGAGGGGAGGCTCGGAGGT GCAGCTTCAGCAGTCT			P3 (UCHT1 VL5)	
SEQ ID NO. 10	GCAAGCTTGAAGACTGTGAGAGTGGTGCCCTTG			P4 (UCHT1 VH3)	Hind III
SEQ ID NO. 11	GTCTCTTCAAAGCTTATTGCC(T)GAGCTGCCCTCCCAA			P5 (Hulgm-CH2)	Hind III
SEQ ID NO. 12	GCATCTAGATCAGTAGCAGGTGCCAGCTGTGT			P6 (Hulgm-CH4)	Xba I
SEQ ID NO. 13	CGGTGACACCATGGAGACAGACACTCCTGTTATGGGT ACTGCTGCTCTGGGTCCA			SP1 (signal seq1)	Sal I
SEQ ID NO. 14	GTACTGCTGCTCTGGGTCCAGGTCCACTGGGGACATCC AGATGACCCAG			SP2 (signal seq2)	

RE: restriction enzyme.

Restriction sites appeared in the primers were underlined and bold.

The primers listed as SEQ ID NO:8 and SEQ ID NO:11 consisted of a mixture of the sequence without the nucleotide(s) in parentheses and the sequence (s) with the nucleotide(s) in parentheses replacing the immediately preceding nucleotide(s) in the sequence.

Expression in COS-7 and SP2/0 cells.

The gene fragment encoding scUCHT1 was then cloned into an expression vector pLNCX [36]. The scUCHT1 gene construct was introduced into COS-7 cells with a
5 calcium-phosphate method [32], and introduced into SP2/0 myeloma cells by electroporation [33]. Cells transfected were selected with 500 μ g/ml G418 (GIBCO/BRL, Gaithersburg, MD, USA) in DMEM medium. The drug resistant transfectants were screened for scUCHT1 secretion by an
10 anti-human IgM ELISA technique. Transfectants secreting scUCHT1 were cloned by limiting dilution.

Two stable clones, COS-4C10 and SP2/0-7C8, which could produce about 0.5 mg/ml scUCHT1 in culture medium,
15 were selected for further evaluation. The culture supernatant of COS-4C10 and SP2/0-7C8 cells was analyzed by immunoblotting using anti-human IgM antibody (Fig. 5). Human IgM antibody was included as a control in the analysis. Under reducing conditions, scUCHT1 produced by
20 COS-7 and SP2/0 cells had a similar electrophoretic mobility to that of the control human IgM heavy chain (75 kDa). Under non-reducing conditions, scUCHT1 from COS-7 cells appeared as a single band of approximately 150 kDa, which was thought to be a homodimer of the single chain
25 antibody. SP2/0 cells mainly produced a protein of similar size with some higher molecular weight products.

In constructing scUCHT1, the domain orientation of sFv, V_H - V_L , which Shu et al. used to V_L - V_H orientation, was
30 changed so that the heavy chain constant domains were linked to the V_H domain. In mammalian cells, secretion of immunoglobulin molecules is mediated by light chain, and free light chain is readily secreted [38]. However, free heavy chain is generally not secreted [39]. In a
35 bacterial expression system, the yield of secreted sFv

with a V_L - V_H domain orientation was about 20-fold more than that obtained with a V_H - V_L domain orientation [40]. It was reasoned that V_L at the NH2-terminal position and V_H linked to heavy chain constant region in scUCHT1 construct might enhance the secretion of this immunoglobulin-like molecule in mammalian cells. In fact scUCHT1 was efficiently produced by both COS-7 and SP2/0 cells. Hollow fiber culture should increase its production. Moreover, scUCHT1, the IgM-like molecule, has a secretory tailpiece with a penultimate cysteine (Cys 575) which is involved in polymerization and also provides retention and degradation of IgM monomers [41-43]. Replacing the Cys 575 with serine might also greatly improve the yield.

scUCHT1 secreted from COS-7 cells was shown to be a divalent form by immunoblotting, suggesting a disulfide bond linkage of two monovalent molecules. The disulfide bond is likely situated between the CH2 and CH3 regions, where the Cys 337-Cys 337 disulfide bond is thought to exist. Cys 337 is believed to be sufficient for assembly of IgM monomers, and was neither sufficient nor necessary for formation of polymers. However, Cys 575 was necessary for assembly of IgM polymers, and Cys 414 was not required for formation of IgM monomers or polymers [44]. This divalent form of the single chain antibody should increase its binding affinity. While scUCHT1 produced from SP2/0 cells was mainly in the divalent form, a small fraction of the antibody had a higher molecular weight, nearly comparable to that of the human IgM pentamer, the natural form of secreted human IgM.

Western blotting analysis of scUCHT1.

scUCHT1 was precipitated from the culture supernatant using goat anti-human IgM-Agarose (Sigma, St. Louis, MO, USA), and separated on 4-20% SDS-PAGE gradient gel under

reducing and non-reducing conditions. The separated proteins were transferred to ProBlott™ membrane (Applied Biosystems, Foster City, CA,

- USA) by electroblotting at 50 volts for 1 hour. The
 5 membrane was blocked and incubated with alkaline phosphatase labeled goat anti-human IgM antibody (PIERCE, Rockford, IL, USA) following the manufacturer's instruction. Color development was carried out with substrate NBT/BCIP (PIERCE).

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Purification of scUCHT1.

- Culture supernatant was mixed with anti-human IgM-Agarose, and incubated at 4°C with shaking overnight, and then the mixture was transferred to a column. The
 15 column was washed with washing buffer (0.01 M Na-phosphate, pH 7.2, 0.5 M NaCl) until the OD280 of flow-through was <0.01. scUCHT1 was eluted with elution buffer (0.1 M glycine, pH 2.4, and 0.15 M NaCl). The fractions were neutralized with 1 M Na-phosphate (pH 8.0)
 20 immediately, and then concentrated and dialyzed against PBS.

Competitive binding assay.

- The parental antibody UCHT1 was iodinated using
 25 Bolton-Hunter Reagent (NEN, Wilmington, DE, USA) as described previously [34]. The ¹²⁵I-labeled UCHT1 was used as tracer and diluted with DMEM medium to 0.3-0.6 nM. UCHT1 and the purified scUCHT1 from COS-7 and SP2/0 transfectant cells were used as competitors. Human CD3
 30 expressing Jurkat cells were suspended in DMEM medium (2 x 10⁷/ml). 50 µl of such cell suspension (1 x 10⁶) was incubated with 50 µl diluted tracer and 50 ml diluted competitors on ice for 2 hours. Afterwards, cells were pelleted, and counted in a gamma counter. Results were

expressed as a percentage of the ^{125}I -UCHT1 bound to cells in the absence of competitors (Fig. 6).

scUCHT1 from both COS-7 and SP2/0 cells could specifically inhibit the binding of ^{125}I -UCHT1 to Jurkat cells in a dose dependent way. As the concentration of the competitors (UCHT1, scUCHT1 from COS-7 and SP2/0 cells) increased from 1 to 100 nM, the tracer (^{125}I iodinated UCHT1) bound to Jurkat cells decreased from 80% to nearly 0%. No significant difference was observed among the affinity curves of UCHT1 and scUCHT1 from COS-7 and SP2/0 cells. This indicates that the engineered antibody scUCHT1 has nearly the same affinity as UCHT1. Moreover, scUCHT1 contains human IgM constant region, and is expected to be less immunogenic than UCHT1. The degree of its immunogenicity might vary due to the murine variable region of scUCHT1. Humanized variable regions by CDR-grafting or human variable regions can be used to further reduce its immunogenicity [31].

20

T-cell proliferation assay.

T-cell proliferation in response to UCHT1 and scUCHT1 was tested on human PBMCs from a healthy donor (Fig. 7). Human peripheral blood mononuclear cells (PBMCs) were isolated from blood of a healthy adult by density centrifuge over Ficoll-Hypaque gradient [34]. The PBMCs were resuspended in RPMI 1640 supplemented with 10% FCS and aliquoted to 96-well U-bottom plates at 5×10^4 cells/well. Increasing amounts of anti-CD3 antibodies (UCHT1, scUCHT1) were added. After 72 hours of culture at 37°C in a humidified atmosphere containing 5% CO_2 , $1 \mu\text{Ci}$ [^3H]thymidine (NEN) was added to each well. 16 hours later, cells were harvested and [^3H]thymidine incorporation was counted in a liquid scintillation counter.

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The parental antibody UCHT1 started to induce proliferation at 0.1 ng/ml, and peaked at 100 ng/ml. A small drop in CPM was observed as the concentration increased to 1,000 ng/ml. However, [³H]thymidine incorporation in PBMCs incubated with scUCHT1 was only slightly increased in the range of 0.1 - 10 ng/ml, and when the concentration was higher than 10 ng/ml, the incorporated counts decreased and were close to 0 counts at 1,000 ng/ml.

10

Measurement of TNF- α and IFN- γ .

TNF- α and IFN- γ productions of human PBMCs induced by UCHT1 and scUCHT1 were measured with ELISA. 4×10^5 PBMCs were cultured with serial dilutions of anti-CD3 antibodies (UCHT1, scUCHT1) in 96-well flat-bottom plates in RPMI 1640 supplemented with 10% FCS. Supernatant was collected at 36 hours for TNF- α and 72 hours for IFN- γ after the start of the culture [35]. TNF- α and IFN- γ were measured with ELISA kits (Endogen Inc. Cambridge, MA, USA) following the manufacturer's instruction.

The native antibody UCHT1 induced production of both TNF- α and IFN- γ in a dose dependent way (Fig. 8a and 8b). Higher concentration of UCHT1 induced higher production of TNF- α and IFN- γ . On the contrary, scUCHT1 did not induce secretion of TNF- α at any concentration (Fig. 8a), and inhibited IFN- γ production when its concentration was higher than 0.1 ng/ml (Fig. 8b). At the time of supernatant harvesting, the PBMCs cultured with UCHT1 and scUCHT1 were also checked with trypan blue exclusion test. Cells were shown to be alive in both situations. In TNF- α and IFN- γ ELISA assays, an unrelated human IgM was included and it did not affect the TNF- α and IFN- γ production.

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Anti-CD3 mAbs can induce T cell activation and proliferation both in *in vitro* and *in vivo* situations [45]. Crossing-linking of anti-CD3 antibody between T cells and FcR expressing cells is an essential step in this process [46]. T cell activation therefore reflects an efficient interaction of the mAb with a human FcR. Previous data of *in vitro* study indicated that T cell activation resulted in increased production of TNF- α , IFN- γ , and IL-2 [24]. Human IgG Fc receptors (Fc γ R I, Fc γ R II, Fc γ R III) are distributed on human monocytes, T, B lymphocytes, and NK cells [47]. Fc γ R I and Fc γ R II can recognize both mouse and human IgG. In accordance with the above observation, UCHT1 was potent in induction of T cell proliferation and TNF- α and IFN- γ release. Human IgM Fc receptor (Fc μ R) was reported to be present mainly on a small fraction of B lymphocytes, NK cells, and possibly a helper subset of T lymphocytes [47, 48]. Pentamer form of IgM and an intact CH₃ domain are required for optimal binding to Fc μ R. Monomeric or dimeric subunits of IgM are less efficient in binding to Fc μ R [49, 50]. Cross-linking of IgM to Fc μ R on T cells inhibited the mitogen-induced T cell proliferation, and Fc μ R may function as a negative signal transducing molecule [51, 52].

Therefore, it can specifically bind to human CD3 molecule and Fc μ R. It is conceivable that scUCHT1 can cross-link human B and T cells, and possibly T and T cells. In an *in vitro* assay, scUCHT1 from both COS-7 and SP2/0 cells had little effect in the T cell proliferation assay at low concentrations (below 10 ng/ml), and became inhibitory as the concentration increased. In accordance with these results, scUCHT1 did not induce TNF- α production and even inhibited the basal yield of IFN- γ .

The present chimeric anti-CD3 single chain antibody scUCHT1 possesses high human CD3 binding ~~specificity~~ ^{specificity} and affinity, and does not induce T cell proliferation and cytokine release. Moreover, it has a human IgM Fc fragment, which should decrease the possibility of inducing human anti-mouse antibody response. Thus, scUCHT1 can be used for clinical immunosuppressive treatment.

EXAMPLE 11

Cloning the full-length of DT gene for the construction of DTM2.

Corynebacteriophage beta (*C.diphtheriae*) tox 228 gene sequence was from genebank. (*Science* 221, 885-858, 1983). The sequence is 2220 bp. There are 300 bp of 5' untranslated region (1 to 300) including the promoter sequence around (-180 to -10), 1682 of coding region (301-1983) including signal peptide (301 to 376), A chain (377 to 955) and B chain (956 to 1983), and 3' untranslated region (1984 to 2220).

The full-length DT was amplified in two fragments.

The pelB leader sequence (ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTGCGCT GCC CAA CCA GCG ATG GCC 3') SEQ ID NO:1) was added to the 5' end of the DT coding ~~sequence~~ ^{sequence} to all the constructs during polymerase chain reaction by primer EcosingnalDT-1 and EcosingnalDT-2. The upstream fragment of 311 bp (from position 301 to 546 bp) was amplified by oligo EcosingnalDT-2 and p546R with CRM9 DNA as a template and the ~~downstream~~ ^{downstream} fragment of 1471 bp was amplified by p514S and p1983R with the DTM1 DNA as template. Then, the combined PCR product of ~~full-length~~ ^{full-length} DT was amplified with

primer EcosingnalDT-1 and p1983R. As a result, the amplified DT coding sequence (position 376 to 1983bp) acquired the pelB leader sequence added to the 5' end and contains the two mutant sites [(508 Ser to Phe) and (525 Ser to Phe)] as DTM1 does.

Primers:

EcosingnalDT-1 5' ATG AAA TAC CTATTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC GCT GCC CAA 3' (SEQ ID NO:2)
 10 EcosingnalDT-2 5' GGA TTG TTA TTA CTC GCT GCC CAA CAA GCG ATG GCCGGC GCT GAT GATGTT GTT GAT TC 3' (SEQ ID NO:3)
 p546R: 5' CGGTACTATAAACTCTTTCCAATCATCGTC 3' (SEQ ID NO:4)
 p514S: 5' GACGATGATTGGAAGAGTTTTATAGTACCG 3' (SEQ ID NO:5)
 p1983R: 5'AGATCTGTCTGA/CTCATCAGCTTTTGATTCAAAAATAGCG 3'
 15 (SEQ ID NO:6).

A mutant residue was introduced at position 52. The glycine (GGG) at position 52 wild type DT was substituted by glutamic acid (GAG). The two primers p546R and p514S
 20 carried the mutant codon (GGG to GAG). The PCR products of these two primers contained the substituted codon (GAG) instead of codon GGG. The jointed double stranded DNA of the two fragments (1683bp) were cloned into pET 17b by restriction site NdeI and BamHI.

25

The data show that anti-human blocking antibodies are specifically directed at the toxin C-terminus. Although a specific sequence derived from the UCHT1 VLVH regions is described, anyone skilled in the art could make sequence
 30 variations in VLVH domains which can be designed to increase the affinity of the sc-anti-CD3-antibody conferring a more favorable therapeutic ratio to fusion immunotoxins using this derivative. Such modifications are within the scope of the present teaching. The
 35 disadvantage of the monovalent antibody VLVH construct, is

that it has a lower affinity for T cells compared to the chemically coupled conjugate which utilizes a divalent antibody.

5 These are believed to be the first instances of a sc anti-CD3 antibodies. IgM was chosen since very few B cells or macrophages contain IgM Fc receptors. (Binding of immunotoxin to cells other than T cells reduces the specificity of the anti-T cell immunotoxin and this
10 situation is purposefully avoided). However, using a bacterial expression system no carbohydrate is attached to the antibody which also eliminates Fc receptor binding. Thus, substituting other human IgG constant domains would be a routine modification and should be claimed.

15 A variety of divalent fusion protein immunotoxins are provided. These have been expressed in *E. coli*, and Western blots of reduced and non-reduced SDS gels confirm that most of the immunotoxin is secreted as the dimeric
20 (divalent) species (Fig. 9). The position of the toxin has been varied in an attempt to minimize steric hindrance of the divalent antibody site, yet provide the best interactions with the CD3 receptor to facilitate toxin translocation across the membrane. Fig. 10 diagrams
25 PCR amplification. Figs. 11 and 12 show two different clones expressing divalent immunotoxin fusion proteins cartooned in Figs. 13 and 14, respectively. Another variation is shown in Fig. 15. The clone producing this consists of a clone constructed by using the single chain
30 antibody followed by a stop codon and the single chain immunotoxin, all under one promotor (Better et al. *Proc. Natl. Acad. Sci.* 90:457-461, January 1993). After secretion and oxidation of the interchain disulfide, 3 species are present: sc divalent antibody, divalent fusion
35 immunotoxin, and a divalent sc antibody containing only

one toxin. This species is isolated by size separation and is the species cartooned in Fig. 15. The advantage of this species is that steric hindrance to the divalent antibody domains is limited by the presence of only one toxin domain. Other variations are routine to construct given the methods described ^{herein} ~~herin~~ and in the art. Those diagramed are considered to be the most likely to exhibit divalent character. Numerous orientations of toxin relative to antibody domains can be made and many are expected to be effective.

In addition, the length of the toxin C-terminus has been varied to provide optimization between two competing functions. The numbers after DT refer to the number of amino acid residues counting the amino terminus of the toxin A chain as 1. The full length toxin is called DTM1 and was provided by Dr. Richard Youle NINDS, NIH (Nicholls et al. *J. Biol. Chem.* 268(7):5302-5308, 1993). It has point mutations S to F at positions 508 and 525. This full length toxin mutant has the essential mutation of CRM9, S to F at 525 which reduces binding to the DT receptor by 3-4 logs without abolishing the translocation function. The other mutation S to F at 508 has been added because of previous restrictions on cloning mutant DT that can revert to wild type toxin with a minimum lethal dose of 0.1 microgram/kg by means of a single base pair reversion. Other mutations can be routinely made in the C terminus to perform this function (Shen et al. *J. Biol. Chem.* 269(46):29077-29084, 1994). They are: F530A; K526A; N524A; V523A; K516A Y514A. A clone having a single point mutation in DT reducing toxicity by 10-100 fold can be made providing that the clone contains an antibody fragment fusion protein, because chemical conjugation of antibody to DT has been shown to reduce systemic wild type toxin toxicity by 100 fold (Neville et al. *J. Biol. Chem.*

264(25):14653-14661, 1989). Therefore, the present invention provides a full length mutant DT sequence with the 525 S to F mutation alone as well as those listed above. These same mutations are also contemplated for the B chain mutant site in DTM2 and can be made similarly. Previous data with chemical conjugation has shown that the longer the C-terminus the better the translocation function (Colombatti et al. *J. Biol. Chem.* 261(7):3030-3035, 1986). However, the shorter the C-terminus the less effect of circulating anti-toxin blocking antibodies (Example 2). Since patients have different levels of blocking antibodies which can be measured (see toxicity assay in Example 2), the optimal immunotoxin can be selected for individual patients. scUCHT1 fusion proteins with DTM1 and DT483 (see Fig. 16), DT390 (Fig. 17) and DT370 (Fig. 18) have been cloned and expressed in *E. coli*. Each of these variations as well as the divalent scUCHT1 fusion proteins using each of these toxin domains are provided.

20

The present invention provides an improvement on CRM197 (a non-toxic toxin mutant described in U.S. Serial No. 08/034,509, filed September 19, 1994) referred to herein as DTM2. DTM2 has the same mutation as CRM197 plus two mutations in the C-terminus which block binding (see sheet and Fig. 9). This is expected to reduce the likelihood of immune complex disease which could result when CRM197 becomes bound to cells and then is further bound by circulating antitoxin. Kidneys are particularly susceptible. DTM2 can not bind to cells thereby lessening the possibility of tissue damage. In addition DTM2 is made for high level production by including the pelB secretory signal for production in *E. coli* or a iron independent mutated promoter DT sequence cloned from CRM9 DNA for production in *C. diptheriae*. The essential

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feature of DTM2 is the S to F mutation at 525 and the G to E mutation at 52, and a construct containing these two mutations is provided.

5 All of the constructs reported here can be expressed in *E. coli* using *pelB* signal sequences or other appropriate signal sequences. Expression can also be carried out in *C. diphtheriae* using appropriate shuttle vectors (Serwold-Davis et al. *FEMS Microbiol. Letters* 10 66:119-14, 1990) or in protease deficient strains of *B. subtilis* and using appropriate shuttle vectors (Wu et al. *Bio. Technol.* 11:71, January 1993).

15 Throughout this application various publications are referenced by numbers within parentheses. Full citations for these publications may be found at the end of the specification immediately preceding the claims. Also, 20 some publications mentioned hereinabove are hereby incorporated in their entirety by reference. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which 25 this invention pertains.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a 30 reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

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